THE X PROTEIN OF HEPATITIS B VIRUS INHIBITS APOPTOSIS IN HEPATOMA CELLS THROUGH ENHANCING THE METHIONINE ADENOSYLTRANSFERASE 2A GENE EXPRESSION AND REDUCING S-ADENOSYL-METHIONINE PRODUCTION

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Running head: HBx activates MAT2A and inhibits apoptosis in hepatoma cells

The X protein (HBx) of hepatitis B virus (HBV) is involved in the development of hepatocellular carcinoma (HCC) and methionine adenosyltransferase 2A (MAT2A) promotes the growth of liver cancer cells through altering S-adenosyl-methionine (SAM) homeostasis. Thus, we speculated that a link between HBx and MAT2A may contribute to HCC development. In this study, the effects of HBx on MAT2A expression and cell apoptosis were investigated and the molecular mechanism by which HBx and MAT2A regulate tumorigenesis was evaluated. Results from immunohistochemistry analyses of 37 pairs of HBV-associated liver cancer tissues/corresponding peritumor tissues showed that HBx and MAT2A are highly expressed in most liver tumor tissues. Our in vitro results revealed that HBx activates MAT2A expression in a dose-dependent manner in hepatoma cells and such regulation requires cis-regulatory elements NF-κB and CREB on MAT2A gene promoter. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) further demonstrated that HBx facilitates the binding of NF-κB and CREB to MAT2A gene promoter. In addition, over-expression of HBx or MAT2A inhibits cell apoptosis, while knock-down of MAT2A expression stimulates apoptosis in hepatoma cells. Furthermore, we demonstrated that HBx reduces MAT1A expression and SAM production, but enhances MAT2β expression. Thus, we proposed that HBx activates MAT2A expression through NF-κB and CREB signaling pathways to reduce SAM production, inhibit hepatoma cell apoptosis, and perhaps enhance HCC development. These findings would provide new insights into our understanding the molecular mechanisms underlines the effects of HBV infection on the production of MAT2A and the development of HCC.

INTRODUCTION

Hepatitis B virus (HBV) infection can cause severe liver diseases including chronic
hepatitis and hepatocellular carcinoma (HCC) (1). Such infection remains a major health problem with 2 billion people infected worldwide. Among them, 400 million are chronically infected (2). However, the complex mechanism by which HBV infection leads to the development of HCC remains largely unclear.

It has been reported that the X protein (HBx) of HBV plays a crucial role in hepatocarcinogenesis (3). HBx is a multifunctional protein that activates many viral and cellular genes, modulates cellular signal transduction pathways, and regulates cell proliferation and apoptosis (4). Many studies have demonstrated that HBx regulates viral gene expression by transactivating the enhancers of HBV and also mediates the expression of cellular genes in infected cells to facilitate tumorigenesis (5-8). Several responsive elements are involved in the transactivation of HBx, including AP-1, NF-κB, and HIF-1. HBx is also directly interact with components of the basal transcription machinery, such as ribosome binding protein 5, TATA-binding protein, and the transcriptional activator CREB/ATF to regulate gene expression (9-11). These interactions provide molecular mechanisms by which HBx regulates gene transcription, modulates cell proliferation and apoptosis, and stimulates the development of HBV-associated HCC.

Apoptosis plays an important role in the progress of liver diseases, since it goes through various extrinsic or intrinsic pathways with activation of caspases and the possible involvement of mitochondria alternation. Several reports have suggested that HBx can also regulate apoptotic pathways, providing additional potential mechanistic link between the function of HBx and the development of HBV-associated HCC (12,13).

S-adenosylmethionine (SAM), a principal biological methyl donor, is synthesized from methionine and ATP in a reaction catalyzed by methionine adenosyltransferase (MAT). In hepatocytes, the levels of SAM are at high in quiescent and low in proliferating hepatocytes depending on the differentiation status of the cells (14). SAM not only controls liver growth, but also regulates cell apoptosis and its homeostasis in the liver influences MAT activities (15).

Two MAT-encoding genes (MAT1A, MAT2A) are found in the cells. MAT1A gene encodes for the α1 subunit, consisting of a dimer (MAT III) or a tetramer (MAT I), and is expressed in adult quiescent hepatocytes. MAT2A gene encodes for a catalytic subunit (α2), consisting of a native MAT isozyme (MAT II), and is expressed in proliferating liver, dedifferentiating cells, and cancer (15). MAT expression is switched from MAT1A to MAT2A during liver malignant transformation, and such alteration plays an important pathogenetic role in facilitating liver cancer growth (16-18).

Previous studies showed that activation of MAT2A expression stimulates the growth and inhibits apoptosis of cancer cells by changing SAM homeostasis (17,19). HBx has been strongly implicated in tumor cell proliferation and apoptosis during hepatocarcinogenesis. This raised the question of whether HBV infection can activate the expression of MAT2A, resulting in the stimulation of tumor cell proliferation. However, the role of HBx in the transcription of MAT2A has not been investigated. In this study, we explored the possibility of a cross-talk between HBx and MAT2A and investigated the molecular mechanism underlines the effects of HBx on MAT2A expression and tumorigenesis. Our results demonstrated that HBx activated MAT2A expression through NF-κB and CREB signaling pathways, resulting in the decrease of SAM production and the inhibition of hepatoma cell apoptosis.

**EXPERIMENTAL PROCEDURES**
Patients and tissue specimens- Thirty seven cases of HBV-associated HCC collected from Zhongnan Hospital, Wuhan University, between January 2008 and January 2009. No chemotherapy or radiation therapy was instituted before tumor excision. Both the tumors and corresponding peritumoral non-cancerous tissues for each case were selected. All patients were tested positive for HBV surface antigen in serum. Matched normal human liver tissues were obtained from liver trauma patients undergoing the partial hepatectomy. Informed consent in writing was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the local ethics committee. No donor organs were obtained from executed prisoners or other institutionalized persons.

Immunohistochemistry- Representative tissues including both HCC tissues and adjacent non-tumorous liver tissues were selected and sectioned in 4-µm thickness. The tissue samples were fixed by immersion in buffered formalin and embedded in paraffin according to standard procedures. Sections were processed in 0.05 M citrate buffer (pH6.0) and heated in a microwave oven for 10 min for antigen retrieval. Sections were then incubated with the primary antibodies for 60 min at room temperature. All specimens stained for HBx and MAT2A were scored by two independent investigators who were blinded to the tested groups. HBx and MAT2A immunostainings were scored by the percentage of total cells that were positive in the cytoplasm. Slides were graded as: - (0 to 10% cells stained), + (10 to 50% cells stained), ++ (>50% cells stained).

Plasmid construction and cell culture- A 986-bp promoter construct of MAT2A gene, corresponding to the sequence from -951 to +35 (relative to the transcriptional start site) of the 5’-flanking region of the human MAT2A gene, was generated from human genomic DNA by polymerase chain reaction (PCR) using F1 (5’-GAAGGTACCCACGGGCAAGGACGGACTTTGGGAG-3’) and R1 (5’-TCCACGCGTAACGCGTGGAGCTTGGAGCT-3’) as forward and reverse primers carrying the Kpnl and MluI sites at the 5’ and 3’-ends, respectively. The PCR product was cloned into Kpnl and MluI sites of the pGL3-Basic vector. The resulting construct was confirmed by DNA sequencing. The 5’-flanking deletion constructs of the MAT2A promoter, (-548/+35) MAT2A, (-320/+35) MAT2A, and (-108/+35) MAT2A were similarly generated by PCR using the (-951/+35) MAT2A construct as a template. The forward primers were F2 (5’-TCAGGTACCGCAGCAAAAAACCTCCGCGATTCAC-3’), F3 (5’-GTAGGTACCGGTCTCTGGAGGGCGATTGCCA-3’), and F4 (5’-TATGTACCCGCAGCCTGCTACGTAGAA-3’). Site-directed mutagenesis of two NF-κB sites and one CREB site were done by multiple rounds of PCR using the (-548/+35) MAT2A construct as a template and appropriate primers with altered bases. The primers used to make the CREB mutant were: F5 (5’-AGAGCAATCCCCCgtATCTCCTCAG-3’) and R2 (5’-GCAATCCCCGCATCTCAGAtACC-3’). For mutating the NF-κB1 site, the primers were: F6 (5’-GGACGCtACCTCatGGAaGCTATC-3’) and R3 (5’-AAAGCGACTGGaGCTTGgTGGA CCG-3’). For mutating the NF-κB2 site, the primers were: F7 (5’-TAGCTGAAagGTCTCCTGGAtGGCCCG-3’) and R4 (5’-GCCCAGTGATgTATGAGCtGCGCCC-3’). The first two rounds of PCR generated two fragments of DNA, which were gel-purified and used as the templates for a third PCR with F2 and R1 primers. Single and double mutants were also ligated into the luciferase expression vector pGL3-Basic and verified by sequence analysis. The cell lines, including the human normal liver cell line L02 and the hepatoma cell lines
BEL-7404 and HepG2 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in the recommended media supplemented with 10% (vol/vol) fetal bovine serum, 100 U/ml penicillin, and streptomycin at 37°C in an incubator with 5% CO₂.

**Transfection and luciferase reporter assays**- All transfections were performed using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer’s protocol. For the luciferase assay, the cells were transiently co-transfected with the pRL-TK plasmid (Promega, Madison, WI, USA) containing the Renilla luciferase gene, which is used for internal normalization, and various constructs containing different lengths of the MAT2A 5’-flanking region. After 48hrs post-transfection, cell lysates were prepared, and the luciferase activity was measured by using a luciferase assay system (Promega, Madison, WI, USA). Cell lysates (10 μl) and luciferase assay substrates (100 μl) (Promega, Madison, WI, USA) were mixed, and fluorescence intensity was detected by a luminometer (Bio-Rad, Hercules, MA, USA). Assays were performed in triplicate and expressed as means ±SD relative to vector control as 100%. All transfections were performed three times.

**Western blotting**- Nuclear and cytoplasmic protein extracts were prepared from transfected cells and used for western blot analysis using rabbit polyclonal antibodies. Protein (30 μg) from each sample was examined by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad, Hercules, MA, USA). Nitrocellulose membranes were subsequently subjected to western blot analysis using the ECL Western Blotting Kit according to procedures described by the manufacturer (Amersham, Arling Heights, IL, USA).

**MATII-specific enzyme activity assay**- MAT II-specific activity assays were performed as described previously (15). Protein extracts were obtained from transfected cells by sonication and then centrifugation at 13000 x g for 15 min. Protein concentrations were determined by the Bradford (18) method, and 250 μg was added to the reaction mixture containing 80 mM Tris-HCl (pH7.4), 50 mM KCl, 40 mM MgCl₂, 5 mM ATP, 10 mM dithiothreitol, 0.5 mM ethylenediaminetetraacetic acid, 50 μM methionine and 0.3 μCi L-[Methyl-³H] methionine. The mixture was applied to a phosphocellulose paper disc (HA 0.45-μm, Millipore) and placed on a filtering system for washing. The disc was added to 10 ml of ScintiVerse E for scintillation counting using a Beckman model LS6000TA Liquid Scintillation Counter (Beckman Instruments, Fullerton, CA, USA). MAT II activity was reported as nmol SAM formed per mg protein per 40 min. SAM and SAH contents were determined by reverse-phase high performance liquid chromatography (HPLC), as described previously (19).

**Quantification of the levels of SAM and SAH by HPLC**- SAM and SAH contents were determined by reverse-phase high performance liquid chromatography (HPLC), as described previously (19). The SAM and SAH standards were dissolved in distal water at a concentration of 1 mmol/L and then diluted with 0.4 mol/L HClO₄ to the final concentrations used for HPLC analysis. 25 μl of standard solution containing 50-11000 pmol was injected into HPLC for preparation of standard curve. The homogenate was centrifuged at 1,000g for 5 min (Beckman glycoprotein receptor centrifuge). The pellet was resuspended in 0.5 M perchloric acid (PCA) and centrifuged at 1,000g for 15min. The aqueous layer was quantitatively removed and neutralized with 3 M KOH. SAM and SAH were determined in the neutralized PCA extracts.
by HPLC (series 410 LC pump, Perkin Elmer) with a LC-90 UV detector and a LC-100 integrator (Perkin Elmer) using a Partisil SCX 10-μm column (25 3 0.44 cm ID; Whatman). SAM and SAH were identified by measuring absorbance at 254 nm at a sensitivity scale of 0.01. The amount of SAM and SAH in each sample was calculated from standard curves of SAM and SAH prepared at the same time as the samples. The identity of SAM and SAH peaks was also confirmed by spiking the sample with known standards.

Electrophoresis mobility shift assay (EMSA)- Nuclear extracts were prepared from HepG2 cells. Probes were generated by annealing single-strand oligonucleotides containing the cognate promoter regions of the MAT2A gene and labeling the ends with [γ-32P]ATP using T4 polynucleotide kinase (TaKaRa). For the CREB site, the oligonucleotide sequences were: 5′-AGAGCAATCCCCACGTCTCCTCGC-3′ and 5′-GCGAGGAGACGTGGGGGATTGCTCT-3′. For the NF-κB site, the oligonucleotide sequences were: 5′-TCTGGAGGGCCGGATTGCCACGGCA-3′ and 5′-TGGCCGTGGCAATCCGGCCCTCCAGA-3′. The MAT2A cis-regulatory motifs of CREB and NF-κB were analyzed. EMSAs were performed with 4 μg of nuclear extract in binding buffer (20 mM Hepes, pH7.9, 0.1 mM EDTA, pH8.0, 75 mM KCl, 2.5 mM MgCl2, and 1 mM DTT) containing 1 μg of poly(dI-dC). To assure the specific binding of transcription factors to the probe, unlabeled double-stranded oligonucleotide competitors were pre-incubated at a 50-fold molar excess for 10 min prior to probe addition. For super-shift experiments, 2 μg of purified polyclonal antibody directed against NF-κB subunit p65 (anti-p65), CREB (anti-CREB) or IgG was incubated with nuclear extracts on ice for 30 min before being added to the binding buffer. Samples were then separated by electrophoresis on 5% nondenaturing polyacrylamide, 0.25×Tris/borate/EDTA (TBE) gels, and the gels were dried and subjected to autoradiography.

Chromatin immunoprecipitation assay (Chip)- HepG2 cells were transfected with relevant plasmids and then cross-linked using 1% formaldehyde at 37°C for 10 min. After washing with PBS, cells were resuspended in 300 μl lyses buffer (50 mM Tris, pH8.1, 10 mM EDTA, 1%SDS, 1 mM PMSF). DNA was sheared to small fragments by sonication. The supernatants were pre-cleared using herring sperm DNA/protein G-Sepharose slurry (Sigma-Aldrich, USA). The recovered supernatants were incubated with antibody directed against NF-κB (anti-p50, p65, p52, and RelB) and CREB (anti-CREB1 and CREB2) or an isotype control IgG for 2hrs in the presence of herring sperm DNA and protein G-Sepharose beads. The immunoprecipitated DNA was retrieved from the beads with 1% SDS and 1.1 M NaHCO3 solution at 65°C for 6h. DNA was then purified using a PCR purification kit (Qiagen, USA), and PCR was done on the extracted DNA using MAT2A promoter-specific primers. For Chip1, the primer was: 5′-GGACGCGACCTCCGGGAAAGCTATC-3′ and 5′-GGACGCGACCTCCGGGAAAGCTATC-3′. For Chip2, the primer was: 5′-GGACGCGACCTCCGGGAAAGCTATC-3′ and 5′-GGACGCGACCTCCGGGAAAGCTATC-3′. The MAT2A cis-regulatory motifs of CREB and NF-κB were analyzed. EMSAs were performed with 4 μg of nuclear extract in binding buffer (20 mM Hepes, pH7.9, 0.1 mM EDTA, pH8.0, 75 mM KCl, 2.5 mM MgCl2, and 1 mM DTT) containing 1 μg of poly(dI-dC). To assure the specific binding of transcription factors to the probe, unlabeled double-stranded oligonucleotide competitors were pre-incubated at a 50-fold molar excess for 10 min prior to probe addition. For super-shift experiments, 2 μg of purified polyclonal antibody directed against NF-κB subunit p65 (anti-p65), CREB (anti-CREB) or IgG was incubated with nuclear extracts on ice for 30 min before being added to the binding buffer. Samples were then separated by electrophoresis on 5% nondenaturing polyacrylamide, 0.25×Tris/borate/EDTA (TBE) gels, and the gels were dried and subjected to autoradiography.

Statistical analysis- The data have been presented as the mean ± standard deviation (X ± SD). The significance of the differences was determined by Student’s t-test. The correlations between the expression of HBx and MAT2A were analyzed by X2 test or Fisher exact test. Spearman rank correlation test was used to determine correlations between the variables. Differences were considered statistically significant for P < 0.05. All statistical analyses were performed using professional statistical software (SPSS 15.0 for Windows, SPSS Inc., Chicago, IL, USA).
RESULTS

MAT2A expression is correlated with HBx expression in liver cancer tissues. The expression status of MAT2A and HBx were examined by immunohistochemical staining of 37 pairs of HBV-associated liver tumor tissues/corresponding peritumoral tissues using antibodies to HBx and MAT2A, respectively (Table 1). Results showed that MAT2A was not detected in normal liver tissue (Fig. 1Aa), expressed at high level in HBV-associated liver tumor tissues (Fig. 1Ab), and produced at lower level in the corresponding peritumoral tissues (Fig. 1Ac). Similarly, HBx was not detected in normal liver tissues (Fig. 1Ad), expressed at high level in liver tumor tissues (Fig. 1Ae), and produced at lower level in peritumoral tissues (Fig. 1Af).

The potential correlation between the expression status of MAT2A and HBx was further analyzed (Table 1 and Fig. 1B). Results indicated that among the 19 liver tumor tissues with high levels of HBx, most (16) of them (84.2%) expressed high levels of MAT2A and the remaining 3 (15.8%) expressed low levels of MAT2A. However, among the 16 liver tumor tissues with low levels of HBx, most (11) of them (68.7%) expressed low levels of MAT2A, and the remaining 5 (31.3%) expressed high levels of MAT2A. In addition, in the 2 cancer tissues with no detectable HBx, MAT2A was also expressed at low level. These results demonstrated that there is a correlation between HBx expression and MAT2A expression with a statistical significance (P=0.0074, chi-square test; P=0.0058, Fisher two-tailed exact test).

To further confirm the expression status of MAT2A and HBx, 7 liver tumor tissues (samples no. 1 to 7) were selected for western blot analyses using antibodies to the two proteins, respectively (Fig. 1C). Results indicated that MAT2A was detected at high level in liver tumor tissues with high level of HBx (Fig. 1C, lanes 1, 2, 3, 5, and 7) and detected at low level in liver tumor tissues with lower level of HBx (Fig. 1C, lanes 4 and 6). These results further demonstrated that a correlation is indeed existed between HBx expression and MAT2A expression in liver tumor tissues.

HBx induces MAT2A expression and enhances MAT II enzyme activity- To determine the effects of HBV on the expression of MAT2A, the levels of MAT2A mRNA and MAT II protein were detected in HepG2 and HepG2.2.15 cells by RT-PCR and western blot analyses, respectively. Results showed that the levels of MAT2A mRNA (Fig. 2A) and MAT II protein (Fig. 2B) were higher in HepG2.2.15 cells than that in HepG2 cells. Since HepG2.2.15 cells, but not HepG2 cells, carrying an integrated HBV genomic DNA on its chromosome, it is reasonable to believe that HBV was responsible for the enhanced expression of MAT2A gene in HepG2.2.15 cells.

The role of HBV in the regulation of MAT II enzyme activity was also determined by transfecting of HepG2 cells with plasmid (pBlue-HBV containing 1.3-fold HBV genome) or control plasmid (its parental plasmid pBlue-SK) and then MAT II activity was measured at different times after transfection. Results showed that MAT II activities were significantly higher in cells transfected with pBlue-HBV than in untransfected cells or in cells transfected with pBlue-SK (Fig. 2C).

Next, we would like to find out the effects of individual proteins of HBV on the activation of MAT2A expression. HepG2 cells were co-transfected with plasmid pGL3-MAT2A, in which the expression of luciferas gene was under the control of MAT2A gene promoter, along with plasmids (pcDNA-S, pcDNA-preS1, pcDNA-preS2, pcDNA-HBe, pcDNA-HBc,
pcDNA-HBx, and pcDNA-HBp) carrying individual genes of HBV or control plasmid, respectively. Results from luciferase activity assays showed that MAT2A promoter activity was significantly stimulated by HBx, but not by the rest of proteins of HBV (Fig. 2D).

To determine the specificity of HBx in the activation of MAT2A gene promoter, HepG2 cells were co-transfected with pGL3-MAT2A and pcDNA-HBx at different concentrations. Results indicated that the level of luciferase activities were increased as the concentrations of pcDNA-HBx increased (Fig. 2E). To evaluate the expression status of HBx in transfected cells, proteins were detected by western blots using antibodies to HBx or GAPDH (as a control). Results showed that the levels of HBx protein were increased as the concentrations of pcDNA-HBx increased in transfected cells, while levels of GAPDH remained relatively unchanged (Fig. 2E). These results suggested that HBx activated MAT2A expression in a dose-dependent manner.

The roles of individual proteins of HBV in the regulation of MAT II enzyme activity were evaluated. HepG2 cells were transfected with plasmids carrying each of the HBV genes or the control plasmid pcDNA-3.1, respectively. Results from MAT II activity analyses indicated that the enzyme activity was stimulated only by HBx, but not by the rest of viral proteins, S, preS1, preS2, HBe, HBc, or P (Fig. 2F).

**MAT2A gene is expressed in hepatoma cell lines and enhanced by HBx.** To investigate the relevance of MAT2A in hepatoma development, the expression levels of endogenous MAT2A gene were determined in normal hepatic cell line and hepatoma cell lines by RT-PCR and Western blot analyses, respectively. Results showed that MAT2A mRNA (Fig. 3A) and its protein (Fig. 3B) were detected in the two hepatoma cell lines (HepG2 and BEL7404), but not in the normal hepatic cell line (L02), indicating MAT2A gene was expressed specifically in hepatoma cells.

The role of HBx in the regulation of MAT2A expression was then analyzed by measuring its effect on the activity of MAT II enzyme in L02, HepG2, and BEL-7404 cell lines, respectively. Cells from the three cell lines were transfected with plasmid pcDNA-HBx at different concentrations. Results showed that HBx enhanced MAT II activity in all three cell lines (Fig. 3C). To evaluate the expression status of HBx in transfected cells, proteins were detected by western blots using antibody to HBx or to GAPDH. Results showed that the levels of HBx protein were increased as the concentrations of pcDNA-HBx increased in transfected cells, while levels of GAPDH remained relatively unchanged (Fig. 3C). These results demonstrated that HBx induced MAT II enzyme activity in a dose-dependent manner in all three cell lines.

**NF-κB and CRE binding elements are required for HBx-activated MAT2A expression.** In order to define the roles of cis-regulatory elements of MAT2A promoter in response to HBx regulation, a series of truncated mutants of MAT2A promoter were generated (Fig. 4A, left panel). To test the functions of these mutated MAT2A promoters, HepG2 and BEL7404 cells were co-transfected with pcDNA-HBx along with plasmids containing the luciferase reporter gene under the control of wild-type and truncated MAT2A promoters. Results from luciferase activity assays indicated that deletion from nt -951 to -548 had no effect on HBx-induced MAT2A promoter activity, deletion from nt -951 to -320 reduced HBx-mediated MAT2A promoter activity, while deletion from nt -951 to -108 eliminated HBx function in the induction of MAT2A (Fig. 4A, right panel). These results suggested that the sequence between nt -548 and -108 was critical for the activation of MAT2A promoter regulated by HBx.

Analyses of the cis-regulatory elements on
the MAT2A promoter revealed two NF-κB binding sites (NF-κB1, NF-κB2) and one CRE binding site (CRE) in this region. To evaluate the roles of these regulatory elements in the activation of MAT2A promoter regulated by HBx, these cis-regulatory elements were altered by site-directed mutagenesis, respectively (Fig. 4B, left panel). Results from luciferase activity assays showed that NF-κB1 mutation had no affect on HBx-induced MAT2A promoter activity, whereas NF-κB2 or CRE mutations significantly reduced the MAT2A promoter activity regulated by HBx (Fig. 4B, right panel). These results suggested that NF-κB2 and CRE recognition elements are required for the activation of MAT2A expression mediated by HBx.

**HBx facilitates the binding of CREB and NF-κB to the MAT2A promoter**- Since CRE and NF-κB2 regulatory elements are required for the activation of MAT2A expression mediated by HBx, it is reasonable to assume that HBx may enhance the binding of CREB and NF-κB to the MAT2A promoter. To confirm this speculation, electrophoresis mobility shift assays (EMSA) were performed. HepG2 cells were transfected with pcDNA-HBx at different concentrations (Fig. 5A and 5B, lanes 2 to 7) or with the control plasmid (Fig. 5A and 5B, lane 1). The nuclear extracts were prepared from transfected cells and incubated with [32P]-labeled DNA probe. To assure the specific binding of transcription factors to the probe, mutated oligonucleotide (Fig. 5A and 5B, lane 5), non-specific competitor (Fig. 5A and 5B, lane 6), and unlabeled double-stranded oligonucleotide competitor (Fig. 5A and 5B, lane 7) were added prior to the addition of labeled probe. To determine the specific binding of NF-κB and CREB to the promoter, polyclonal antibodies to NF-κB (anti-p65) (Fig. 5A, lane 4) or CREB (anti-CREB) (Fig. 5B, lane 4) were incubated with nuclear extracts before adding the binding buffer, respectively. DNA probes used in this study contained either the CREB element (Fig. 5A) or the NF-κB2 element (Fig. 5B) of the MAT2A promoter.

Results from EMSA using the CREB probe showed that one faint band of protein-DNA complex (shift band) was detected in the absence of HBx (Fig. 5A, lane 1), but the shift band were enhanced in the presence of HBx and the level of shift band increased as the concentration of HBx increased (Fig. 5A, lanes 2 and 3). Moreover, the shift band was eliminated in the presence of mutated oligonucleotide (Fig. 5A, lane 5) and unlabeled double-stranded oligonucleotide competitor (Fig. 5A, lane 7), but not affected by non-specific competitor (Fig. 5A, lane 6). In addition, a specific protein-DNA complex (super-shift band) was detected in the presence of anti-CREB antibody (Fig. 5A, lane 4).

Similar results were observed when the NF-κB2 probe was used. EMSA results showed that the protein-DNA complex (shift band) was enhanced in the presence of HBx (Fig. 5B, lanes 2 and 3), while it was reduced in the presence of mutated oligonucleotide (Fig. 5B, lane 5) and unlabeled double-stranded oligonucleotide competitor (Fig. 5B, lane 7), but did not affected by non-specific competitor (Fig. 5B, lane 6). Again, a specific protein-DNA complex (super-shift band) was detected in the presence of anti-CREB antibody (Fig. 5B, lane 4).

The roles of HBx in facilitating the binding of NF-κB and CREB to the MAT2A promoter in vivo were further confirmed by chromatin immunoprecipitation (Chip) assays. Chromatin fragments were prepared from HepG2 cells transfected with pcDNA-HBx and immunoprecipitated with antibodies against either NF-κB (p50, p65, p52, and RelB) or CREB (CREB1 and CREB2), respectively. The locations of the PCR products are indicated as Chip1, Chip2 under the simplified genomic structures of MAT2A promoter (Fig. 5C). Results of Chip1 showed that PCR products
were only produced from DNA isolated from cells transfected with pcDNA-HBx in the presence of antibodies to CREB1 and to CREB2, but not detected in the presence of control plasmids and antibody to p50, p65, p52, or to RelB (Fig. 5C, left panel). Results of Chip2 showed that PCR products were only detected from DNA isolated from cells transfected with pcDNA-HBx in the presence of antibodies to p50, p65, p52, or to RelB, but not detected in the presence of control plasmids and antibodies to CREB1 or to CREB2. These results indicated that HBx enhanced the binding of CREB and NF-κB to the CREB and NF-κB2 recognition sites in the MAT2A promoter, respectively.

**NF-κB and CREB pathways are involved in HBx-regulated MAT2A expression.** The roles of NF-κB and CREB signal transduction pathways in the activation of MAT2A expression regulated by HBx in hepatoma cells were further evaluated. Inhibitors of the signaling components, including MG132 (inhibitor of NF-κB), PD098059 (inhibitor of ERK), U0126 (inhibitor of MEK1/2), H89 (inhibitor of PKA), SP600125 (inhibitor of JNKMAPK), GF109203 (inhibitor of PKC), LY294002 (inhibitor of PI3K), and SB203580 (inhibitor of p38), were added to the cells transfected with pcDNA-HBx for the screening of the signaling pathway(s) involved in the HBx-activated MAT2A expression. MAT II enzyme activities and MAT2A protein levels were determined 72hrs post-transfection. Results indicated that both MAT II activities and MAT2A protein levels activated by HBx were significantly reduced in the presence of inhibitors, MG132, PD098095, U0126, GF109203, LY294002, and SB203580, indicating signaling components, PI3K, MEK1/2, p38 MAP kinase, ERK, PKC, and NF-κB were involved in HBx-enhanced MAT2A protein expression (Fig. 6A).

In order to confirm the effects of those inhibitors on MAT2A expression were specific to HBx, HepG2 cells without transfected with the HBx expression plasmid were treated with these inhibitors. Results showed that MAT II enzyme activities and protein levels were not affected by the treatment of SP600125 (JNKMAPK), H89 (PKA), and PD098095 (ERK), and slightly reduced by the treatment of LY294002 (PI3K), U0126 (MEK1/2) and GF109203 (PKC), and reduced by the treatment of MG132 (NF-κB) and SB203580 (p38), but such reductions caused by MG132 and SB203580 were not significant (Fig. 6B), indicating ERK, MEK1/2, PKC, and PI3K were not involved in the activation of MAT2A expression, while the endogenous NF-κB and p38 had slightly affects on such activation.

Taken together, the above results demonstrated that the effects of those inhibitors of signaling pathways on MAT II protein expression were specific to HBx.

The roles of NF-κB and CREB signal transduction pathways in the activation of MAT2A expression regulated by HBx in hepatoma cells were also evaluated by the approaches of RNA interference. Cells were transfected with pGL3-MAT2A and pcDNA-HBx and treated with shRNA specific to the signaling components. Results showed that HBx-activated MAT2A promoter activity was reduced in the presence of shRNA specific to RIG-I, MAVS, IRAK2, TRAF6, IKKα, IKKβ, IKKi, p50, and p65, respectively (Fig. 6C). We also demonstrated that MAT II activity (Fig. 6D, top panel) and MAT2A mRNA level (Fig. 6D, lower panel) were reduced by the inhibitor of NF-κB (MG132) in a dose-dependent fashion. In addition, MAT II activity (Fig. 6E, top panel) and MAT2A mRNA level (Fig. 6E, lower panel) were also reduced by shRNA to CREB. These results further demonstrated that the NF-κB and CREB signaling pathways play critical roles in HBx-mediated MAT2A expression in hepatoma cells.
HBx inhibits hepatoma cell apoptosis by activating MAT2A and MAT2β expression and inhibiting MAT1A expression and SAM production. The molecular mechanisms underline the roles of HBx in the inhibition of apoptosis in liver cancer cells are not clear. Considering the facts that HBx stimulates MAT2A expression in human HCC and that activation of MAT2A inhibits apoptosis of liver cancer cells by reducing hepatic SAM levels, we speculated that activation of MAT2A mediated by HBx may link to its anti-apoptotic effect on hepatoma cells due to altered SAM production.

To test this hypothesis, HepG2 cells were co-transfected with a plasmid expressing shRNA specific to MAT2A along with pcDNA-HBx or control plasmid. Changes in nuclear morphology of transfected cells were examined by staining of nuclear DNA with DAPI. Results showed that a small proportion of cells with typical hallmarks of apoptosis, such as nuclear fragmentation and chromatin condensation, were detected in cells transfected with pcDNA-3.1 (Fig. 7Aa). The numbers of apoptotic cells were decreased in the presence of MAT2A (Fig. 7Ab) or HBx (Fig. 7Ac), respectively, indicating MAT2A and HBx had an inhibitory effect on apoptosis in hepatoma cells. In addition, the numbers of apoptotic cells were increased after treated with shRNA specific to MAT2A in the absence (Fig. 7Ad) or presence of HBx (Fig. 7Ae), suggesting anti-apoptotic effect on hepatoma cells was due to the expression of MAT2A.

The rates of apoptosis were further evaluated by flow cytometry analyses of sub-G1 populations of apoptotic cells (Fig. 7B and 7C). Results showed that percentage of apoptotic cells was decreased from 13.07±2.96% in the presence of pcDNA-3.1 (Fig. 7Ba and 7C) to 3.72±0.69% and to 5.24±0.85% in the presence of MAT2A (Fig. 7Bb and 7C) and HBx (Fig. 7Bc and 7C), respectively. However, the percentage of apoptotic cells was increased to 33.21±6.65% and 19.31±5.13% when MAT2A was knocked down by shRNA in the absence (Fig. 7Bd and 7C) and presence (Fig. 7Be and 7C) of HBx, respectively (P<0.01). These results again suggested that the anti-apoptotic effect on hepatoma cells was due to the expression of MAT2A.

Since inhibition of apoptotic cell death in hepatoma cells by MAT2A may be resulted from a reduction of SAM level, we evaluated the changes in the concentrations of SAM under the conditions of knocked-down of MAT2A and over-expression of MAT2A or HBx in transfected cells, respectively. Results showed that SAM levels were slightly reduced when MAT2A or HBx was present, as compared to the control, while increased when MAT2A gene was knocked-down by shRNA in the absence or presence of HBx (Fig. 7D). However, SAH levels were remained relatively unchanged under the same conditions (Fig. 7D).

The preservation of MAT1A expression and MATI/III activity, is a fundamental trait of the healthy and differentiated hepatocyte. Loss of MAT1A expression results in the malignant transformation of the liver, while the replacement MATI/III by MATII appears to confer a growth advantage to the transformed cell. Thus, we further determined the effects of HBx on the expression of MAT proteins (MAT1A, MAT2A, and MAT2β) by western blot analyses. Results showed that in the absence of HBx, MAT1A was highly expressed and MAT2A or MAT2β was not detected, but in the presence of HBx, MAT1A expression was inhibited and MAT2A or MAT2β expression was induced (Fig. 7E).

The effects of HBx on the production of SAM and SAH were also determined. Results indicated that HBX inhibited SAM production in an HBx-dose dependent manner, but not affected SAH production (Fig. 7F). In the afford to analyze the effects of HBx on the levels of SAM, MAT, and SAH, we found that a 30%
reduction in intracellular SAM production was detected in HepG2 cells transfected with 0.25 µg of pcDNA-HBx and a further 50% reduction in SAM production was measured in cells transfected with 0.50 µg of pcDNA-HBx (Table 2). The ratio of SAM:SAH was decreased in parallel with SAM level, whereas SAH and MTA level remained relatively unchanged (Table 2).

These results demonstrated that HBx inhibits hepatoma cell apoptosis by activating MAT2A and MAT2β expression and inhibiting MAT1A expression and SAM production.

**DISCUSSION**

Hepatitis B virus is a major causative agent of acute and chronic hepatitis in humans and is closely associated with the incidence of human hepatocellular carcinoma (HCC) (20). The X protein of HBV is essential for transactivation of the viral and some cellular genes (21,22). Epidemiological and molecular evidence indicates that HBx is involved in the development of primary HCC (23,24). HBx is reported to induce liver cancer growth in transgenic mice and to activate several host genes important for cell proliferation and transformation, such as c-fos, c-myc, and β-interferon. HBx is a trans-activating protein that alters gene expression by interacting with transcription factors or stimulating signaling pathways that promote cell growth and survival. HBx also binds to and inactivates tumor suppressors and senescence-related factors (24,26). However, the mechanisms behind the role of HBx in the regulation of HCC development remain largely unknown.

In this study, we identified MAT2A as a new target of HBx. HBx activates the MAT2A promoter and induces its expression in hepatic cell line (L02) and hepatoma cell lines (HepG2 and BEL-7404) in a dose-dependent manner, indicating HBx is capable of regulating MAT2A expression. To gain insight into the mechanism behind the function of HBx in the regulation of MAT2A expression, a series of mutations of MAT2A promoter were constructed and assayed. Our results indicated that the sequence between nt -548 and -108 of the promoter of MAT2A gene along with NF-κB2 and CREB recognition elements are required for the function of HBx in the activation of MAT2A expression. HBx facilitates the binding of NF-κB and CREB to the MAT2A promoter. In addition, we further confirmed that NF-κB and CREB signal transduction pathways are involved in the activation of MAT2A expression regulated by HBx in hepatoma cells. These results suggested that one of the mechanisms in which HBx regulates HCC development is through activating MAT2A expression.

In mammals, two genes (MAT1A and MAT2A) encode two homologous catalytic subunits of MAT enzyme. Normal liver expresses MAT1A, whereas MAT2A is induced in human hepatocellular carcinomas (HCC) and facilitates cancer cell growth (27,28). Studies have shown that a switch in gene expression from MAT1A to MAT2A in human liver cancer is pathogenetically important, as stimulation of MAT2A expression in cancer cells enhances cell growth through DNA hypomethylation, whereas inhibition of MAT2A expression in hepatoma cells has a reverse phenotype (29,30). Thus, MAT2A functions as a positive regulator in hepatoma growth, suggesting manipulating MAT2A expression may have therapeutic potential for the treatment of hepatoma.

MAT2A is transcriptionally induced in human HCC and in rodents during rapid liver growth and dedifferentiation. Many studies have shown that increased MAT2A expression provides liver cancer cells a growth advantage by inhibiting cellular apoptosis. HBx protein also can inhibit cell apoptosis during the development of HBV-associated HCC. The anti-apoptotic effect shared by HBx and
MAT2A prompted us to examine the effects of HBx on MAT2A activation and cell apoptosis in hepatoma cells. Our results showed that both HBx and MAT2A inhibit cell apoptosis in HepG2 cells. In contrast, knock-down of MAT2A expression by shRNA induces cell apoptosis in hepatoma cell, even in the presence of HBx. These results suggested that MAT2A is a downstream target of HBx and may partially explain the anti-apoptotic effect of HBx on the development of HBV-associated HCC.

However, the roles of HBx and its regulator effect on apoptosis are still controversial, with some studies showing pro-apoptotic effects (31-33). In fact, HBx is known to localize in both the mitochondria and the nuclei of cells and subsequently modulates mitochondrial membrane potential and transcription of certain genes, suggesting that the status and localization of HBx for the regulation of NF-κB activation and apoptosis may be changed according to the processing phase of its functional activity within a cell.

MAT enzyme activity is required for the production of SAM, because SAM is synthesized from methionine and ATP in a reaction catalyzed by the enzyme (34). In normal liver cells, two mechanisms to maintain the high cellular level of SAM: 1) up-regulation of MAT1A expression by SAM with the increase in MAT I/III activity; and 2) the high capacity of MAT I/III to convert dietary methionine and ATP into SAM. Since SAM down-regulates MAT2A expression and inhibits MAT II activity, the contribution of MAT II to the production of SAM is minimal in liver cells (15). SAM has rapidly moved from being a methyl donor to a key metabolite that regulates hepatocyte growth, death, and differentiation (35-38). In hepatocytes, SAM levels are related to the differentiation status of the cells. SAM levels are at high in quiescent and at low in proliferating hepatocytes during liver regeneration. The high level of hepatic SAM is transient. If the high level of hepatic SAM was persisting, it would favor a proliferative phenotype and ultimately the development of HCC (39,40).

Liver injury caused by hepatotoxins or partial hepatectomy initiates a cellular response that involves a vast number of growth factors and cytokines (such as HGF, TNF-α, and IL-6) and generation of oxidative stress (NO and ROS), which leads to the inactivation of MAT I/III and a reduction of hepatic SAM level. This reduction in SAM level induces MAT2A expression and MAT II activity, which results in a new lower steady state level of SAM. The reduced level of hepatic SAM releases the inhibitory effect of this molecule on the proliferative activity of HGF, which facilitates liver regeneration. If the conditions leading to oxidative stress persist (e.g., chronic HBV infection), the hepatic levels of SAM are continuously low, which predisposes the liver to develop steatohepatitis, cirrhosis, and ultimately HCC (39,40). In the cirrhotic liver, MAT1A expression is progressively silenced by a mechanism that involves the methylation of the gene promoter and its association with hypoacetylated histones (15).

In cancerous liver cells, MAT activity is very high when methionine is at low physiological concentrations (50-80 μM). However, in cultured normal rat and human hepatocytes, MAT activity is also very high when methionine concentrations are high (5 mM) (41). This led us to speculate that MAT2β, a regulatory subunit of MAT2A, is activated in response to HBx stimulation. The MATII can reduce the Km for methionine and the Ki for SAM, making the subunit more susceptible to feedback inhibition (42). The steady state SAM level should be lower when the level of MAT2A is higher due to this feedback regulation. Our results showed that a sharp decrease of MAT1A protein levels, and increases of MAT2A and MAT2β protein levels were detected in the
presence of HBx in hepatocytes. In accordance with changes of the expression of MAT genes, the intracellular SAM levels were remarkably decreased in response to HBx stimulation.

SAM not only controls liver growth, but also regulates apoptosis, with an anti-apoptotic role in normal hepatocytes and a pro-apoptotic role in liver cancer cells (43-45). MAT2A inhibits apoptosis of liver cancer cells, contributing to a reduction of steady-state SAM levels. The reduction in hepatic SAM levels can feed into a vicious cycle that favors a switch in MAT expression and liver dedifferentiation (29).

In an afford to investigate the effect of MAT2A on the changes in SAM levels and cell apoptosis regulated by HBx, we demonstrated that the changes in hepatoma cell apoptosis were concomitant with changes in SAM production. Knock-down of MAT2A expression by shRNA stimulated SAM production, and thus abolished the inhibitory effect of HBx on apoptosis in HepG2 cells. These results suggested that MAT2A and SAM act oppositely to the inhibition of apoptosis in hepatoma cells regulated by HBx.

In summary, we demonstrated that the X protein of HBV directly regulates the expression of MAT2A gene in hepatoma cells through enhancing the binding of transcription factors NF-κB and CREB to the promoter of MAT2A gene. We proposed that during HBV infection, the viral protein HBx stimulates the expression of MAT2A gene, resulting in a increase of MAT II enzyme activity, a decrease of SAM production, and the inhibition of apoptotic cell death in cancer cells (Fig. 8). Our results suggested that HBx-induced MAT2A expression may play an important role in HBV-mediated HCC progression, which would provide new insights into our understanding the molecular mechanisms involved in the development of HCC caused by HBV infection.

REFERENCES

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**FIGURE LEGENDS**

Fig. 1. Determination of expression status of MAT2A and HBx in HBV-associated liver tumor tissues by immunohistochemistry analyses.  
A. Representative results of immunohistochemistry analyses of 37 cases of surgically resected HBV-associated HCC tissues and their adjacent non-tumorous liver tissues. (a, d) Normal liver tissues obtained from liver traumas patients undergoing partial hepectomy; (b, e) HBV-associated HCC tissues; (c, f) Peritumoral non-cancerous tissues. (a, b, and c) Immunohistochemistry analyses using antibody to MAT2A; (d, e, and f) Immunohistochemistry analyses using antibody to HBx.  
B. Analyses of the correlation between the expression level of MAT2A and HBx. The levels (high ++; low +; and undetectable -) of HBx in HBV-associated HCC tissues were compared to that of MAT2A, respectively ($P=0.0075$, chi-square test; $P=0.0061$, Fisher two-tailed exact test).  
C. 7 HBV-associated HCC tissues (samples no. 1 to 7) were selected for western blot analyses using antibodies to MAT2A, HBx, and GAPGH proteins, respectively.

Fig. 2. The role of HBx in the regulation of MAT2A expression in hepatoma cells.  
A. RT-PCR analyses of mRNA levels in HepG2 and HepG2.2.15 cells using primers specific to the MAT2A, HBx, and GAPDH gene, respectively.  
B. Western blot analyses of protein expression status in HepG2 and HepG2.2.15 cells using antibodies to MAT2A, HBx, and GAPDH proteins, respectively.  
C. HepG2 cells were transfected with pBlue-SK or pBlue-HBV, respectively, at different times as indicated. MAT II enzyme activities were determined.  
D. HepG2 cells were co-transfected with a reporter plasmid pGL3-MAT2A, in which the luciferase gene is under the control of the MAT2A promoter, along with plasmids (pcDNA-S, pcDNA-preS1, pcDNA-preS2, pcDNA-HBs, pcDNA-HBx, and pcDNA-HBp) expressing each of the HBV proteins, respectively. Relative luciferase activity was determined by standard procedures. Results shown are mean ± S.D. of three experiments performed in duplicate.  
E. HepG2 cells were co-transfected with the reporter plasmid pGL3-MAT2A and pcDNA-HBx at different concentrations. Relative luciferase activity was determined by standard procedures.
procedures. Results shown are mean ± S.D. of three experiments performed in duplicate. The protein levels were determined by western blot using antibodies to HBx and GAPDH, respectively. F. HepG2 cells were transfected with pcDNA3.1 or plasmids expressing each of the HBV proteins, respectively. MAT II enzyme activities were determined 48hrs post-transfection.

**Fig. 3.** Determination of MAT2A expression levels and MAT II activities regulated by HBx. A. RT-PCR analyses of mRNA levels in L02, HepG2, and BEL7404 cells using primers specific to MAT2A or GAPDH gene. B. Western blot analyses of protein levels in L02, HepG2, and BEL7404 cells using antibodies to MAT2A or GAPDH proteins. C. L02, HepG2, and BEL7404 cells were transfected with pcDNA-HBx at 0 μg, 0.25 μg, and 0.5 μg per well. MAT II enzyme activity was measured 48hrs post-transfection. Data are shown as the mean ± S.D. of three independent experiments. The expression status of HBx and GAPDH was also determined using antibodies to the two proteins, respectively.

**Fig. 4.** The requirement of cis-acting elements in the activation of MAT2A gene expression regulated by HBx. A, left panel. Diagrams of reporter constructs containing the luciferase gene under the control of the human MAT2A promoter or its serial deletions. A, right panel. HepG2 and BEL-7404 cell lines were co-transfected with pcDNA-HBx along with the constructed reporter plasmids with wild-type or mutated MAT2A promoters. Relative luciferase activity was determined. The results shown are the means’ ± S.D. of three experiments performed in duplicate. B, left panel. Diagrams of reporter constructs containing the luciferase gene under the control of MAT2A promoter with specific mutations in cis-regulatory elements as indicated. B, right panel. HepG2 and BEL-7404 cell lines were co-transfected with pcDNA-HBx along with these constructed reporter plasmids with wild-type or mutated MAT2A promoters. Relative luciferase activity was determined. The results shown are the means’ ± S.D. of three experiments performed in duplicate.

**Fig. 5.** Analyses of the effects of HBx on the binding of CREB and NF-κB to the MAT2A promoter. A. Analyses of the effect of HBx on the binding of CREB to the MAT2A promoter by EMSA. EMSA was performed with nuclear extracts of HepG2 cells transfected with control plasmid (lane 1) or pcDNA-HBx at different concentrations (lanes 2 to 7). CREB probe was generated by annealing single-stranded and end-labeled oligonucleotides containing the cognate MAT2A promoter region (nucleotides -385/-365). Mutated oligonucleotide (lane 2), non-specific competitor (lane 6), or specific competitor (unlabeled CREB probe, lane 7) were used as a control. For super-shift, antibody to CREB (lane 4) was incubated with nuclear extracts before being added to the reaction. B. Analyses of the effect of HBx on the binding of NF-κB to the MAT2A promoter by EMSA. EMSA was performed with nuclear extracts of HepG2 cells transfected with pcDNA-HBx. NF-κB probe was generated by annealing single-stranded and end-labeled oligonucleotides containing the cognate MAT2A promoter region (nucleotides -296/-284). Mutated oligonucleotide (lane 2), non-specific competitor (lane 6), or specific competitor (unlabeled NF-κB probe, lane 7) were used as a control. For super-shift, antibody to p65 (lane 4) was incubated with nuclear extracts before being added to the reaction. Samples were electrophoresed on 5% non-denaturing polyacrylamide gels and visualized by autoradiography. Arrows indicate the shift bands or supershifted protein-DNA complexes or free probes. C. Determination of the role of HBx in the binding of CREB and NF-κB to the MAT2A promoter by Chip assays. HepG2 cells transfected with pcDNA-HBx (+) or control vector (-) were
lysed and subjected to Chip assays. The exact locations of PCR products of Chip1 and Chip2 are underlined the simplified genomic structures of the MAT2A gene promoter. The results are representatives of 4 independent experiments.

**Fig. 6.** Determination of signaling pathways involved in the activation of MAT2A gene expression regulated by HBx.  
**A.** Analyses of the effects of inhibitors of signaling components on the activation of MAT2A expression regulated by HBx. HepG2 cells were transfected with pcDNA-HBx and treated with LY294002 (10 μM), U0126 (13 μM), MG132 (4 μM), SB203580 (10 μM), GF109203 (1 μM), H89 (10 μM), SP600125 (30 μM), and PD098059 (10 μM), respectively. MAT II enzyme activity was measured and MAT2A protein was determined by western blot analyses 72hrs post-transfection.  
**B.** HepG2 cells were transfected with pcDNA and treated with LY294002 (10 μM), U0126 (13 μM), MG132 (4 μM), SB203580 (10 μM), GF109203 (1 μM), H89 (10 μM), SP600125 (30 μM), and PD098059 (10 μM), respectively. MAT II enzyme activity was measured and MAT2A protein was determined by western blot analyses 72hrs post-transfection.  
**C.** Determination of the effects of shRNAs of signaling components on the activation of MAT2A expression regulated by HBx. HepG2 cells were co-transfected with pMAT2A-Luc and pcDNA-HBx along with plasmids expressing shRNA-control, shRNA-RIG-I, shRNA-MAV, shRNA-IRAK2, shRNA-TRAF6, shRNA-IKKα, shRNA-IKKβ, shRNA-IKKi, shRNA-p50, or shRNA-p65, respectively. Luciferase activity was measured 72hrs post-transfection.  
**D.** The effect of MG132 on the MAT II enzyme activity and MAT2A gene expression regulated by HBx. HepG2 cells were transfected with pcDNA-HBx and treated with MG132 at different concentrations as indicated. MAT II activity was measured (top panel) and MAT2A and GAPDH mRNA levels were determined by RT-PCR (lower panel) 72hrs post-transfection.  
**E.** The effect of shRNA to CREB on the MAT II enzyme activity and MAT2A gene expression regulated by HBx. HepG2 cells were co-transfected with pcDNA-HBx and pshRNA-CREB. MAT II activity was measured (top panel) and MAT2A and GAPDH mRNA were determined RT-PCR (lower panel) 72hrs post-transfection.

**Fig. 7.** The effects of HBx and MAT2A on cell apoptosis and SAM production in hepatoma cells.  
HepG2 cells were plated at density of 2×10⁵ cells/cm².  
**A-B.** HepG2 cells were transfected with pcDNA-3.1 (a), pcDNA-MAT2A (b), pcDNA-HBx (c), pMAT2A-shRNA (d), and pcDNA-HBx plus pMAT2A-shRNA (e).  
**A.** Changes in nuclear morphology of transfected cells were examined by staining of nuclear DNA with DAPI and visualized under fluorescence microscopy.  
**B.** The rates of apoptosis were evaluated by the determination of sub-G1 populations of transfected cells though Guava Nexin-V assays using flow cytometry.  
**C.** The rates of apoptosis were summarized from flow cytometry analyses.  
**D.** The levels of SAM and SAH produced in transfected cells were determined by reverse-phase high performance liquid chromatography (HPLC). Results in C and D are mean of three independent experiments and the bars represent ±SD, significantly different from control by Turkey test.  
**E.** The effects of HBx on the expression of MAT genes. L02 cells were transfected with pcDNA-HBx at different concentration as indicated. The levels of MAT1A, MAT2A, MAT2β, and GAPDH proteins were determined using antibodies to the four proteins respectively.  
**F.** The effects of HBx on the change in SAM homeostasis. L02 cells were transfected with pcDNA-HBx at different concentration as indicated. The levels of SAM and SAH were measured by HPLC. Results were mean of three independent experiments, significantly different from control by Turkey test.
Fig. 8. Proposed model for the role of HBx in MAT2A expression, SAM production, cell apoptosis, and perhaps HCC development.  

A. In normal liver cells, two mechanisms to maintain the high cellular SAM level: ① up-regulation of MAT1A expression by SAM with the increase in MAT I/III activity; and ② the high capacity of MAT I/III to convert dietary methionine and ATP into SAM. Since SAM down-regulates MAT2A expression and inhibits MAT II activity, the contribution of MAT II to the production of SAM is minimal in liver cells. SAM controls liver growth and also regulates apoptosis with an anti-apoptotic effect on normal hepatocytes.  

B. During HBV infection, the viral protein HBx activates MAT2A expression and MAT II activity by enhancing the binding of NF-κB and CREB to MAT2A gene promoter. MAT2A facilitates cancer cell growth through DNA hypomethylation and thus, it functions as a positive regulator in hepatoma growth. In addition, by witch in gene expression, highly expressed MAT2A inhibits MAT1A expression, which is progressively silenced by a mechanism that involves the methylation of MAT1A gene promoter and its association with hypoacetylated histones. As a result, a new lower steady state level of SAM is reached. Such reduction in SAM level releases the inhibitory effect of this molecule on proliferation, which facilitates liver regeneration. If the conditions leading to chronic HBV infection is persistent, MAT2A levels are maintained continuously high and SAM levels are maintained continuously low, which result in the inhibition of apoptotic cell death and predisposes the liver to develop steatohepatitis, cirrhosis, and ultimately HCC.
Table 1. Expression status of HBx and MAT2A in HBV-associated liver tumor tissues and the corresponding peritumoral tissues.

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<tr>
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<td>54</td>
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<td>No</td>
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<td>Gender</td>
<td>Tumor Volume (cm³)</td>
<td>Axis</td>
<td>Viral Type</td>
<td>Stage</td>
<td>Immunohistochemical Staining</td>
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<tr>
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<td>------------</td>
<td>-------</td>
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<td>++</td>
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Tumor volume (cm³) was calculated by the formula: length (cm) × shortest width (cm) × longest width (cm), in which length was the longest axis. Immunohistochemical (IHC) staining was estimated as follows: - (0 to 10% cells stained), + (10 to 50% cells stained), ++ (>50% cells stained).
Table 2. Changes in SAM, MTA, SAH Levels in HepG 2 cells regulated by the different concentration of HBx.

<table>
<thead>
<tr>
<th>HBx (µg)</th>
<th>SAM</th>
<th>MTA</th>
<th>SAH</th>
<th>SAM/SAH</th>
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<tr>
<td>0</td>
<td>0.72±0.16</td>
<td>0.41±0.08</td>
<td>0.35±0.06</td>
<td>2.1±0.05</td>
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<tr>
<td>0.25</td>
<td>0.51±0.12*</td>
<td>0.38±0.05</td>
<td>0.32±0.03</td>
<td>1.6±0.04*</td>
</tr>
<tr>
<td>0.50</td>
<td>0.37±0.09*</td>
<td>0.36±0.06</td>
<td>0.31±0.04</td>
<td>1.0±0.01*</td>
</tr>
</tbody>
</table>

*P < 0.05 verse HBx 0 µg by unpaired Student’s t-test. Results represent means ± SE from four to five separate determinations. SAM, S-adenosyl-methionine; SAH, S-adenosylethionine; MTA, methylthioadenosine.
Figures

Figure 1

Fig. 1A

Normal liver tissues  HBV-associated HCC tissues  Peritumoral non-cancerous

(a)  (b)  (c)

anti-MATIIA antibody

(d)  (e)  (f)

anti-HBx antibody

Fig. 1B

Fig. 1C

MAT2A staining intensity (%)

HBx Staining intensity

Sample 1 2 3 4 5 6 7

MAT2A Protein
HBx Protein
GAPDH Protein
Figure 2

Fig. 2A

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HepG2</th>
<th>HepG2215</th>
</tr>
</thead>
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<tr>
<td>MAT2A mRNA</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>HBx mRNA</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>GAPDH mRNA</td>
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Fig. 2B

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<td>MAT2A protein</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>HBx protein</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
<tr>
<td>GAPDH protein</td>
<td>![Image]</td>
<td>![Image]</td>
</tr>
</tbody>
</table>

Fig. 2C

![Graph](image)

Time after transfection (h)

MAT II activity

untreat

pBlue-SK

pBlue-HBV

Fig. 2D

![Graph](image)

Relative Luciferase Activity (%)
Fig. 2E

Relative Luciferase Activity

0 100 200 300 400 500 600

pcDNA-HBx (µg) 0 0.1 0.2 0.4 0.8

HBx protein

GAPDH protein

Fig. 2F

MAT II activity

0 0.02 0.04 0.06 0.08 0.1 0.12 0.14 0.16 0.18

1 2 3 4 5 6 7 8 9

untreated pcDNA3.1 pcDNA-S pcDNA-preS1 pcDNA-preS2 pcDNA-Hbe pcDNA-Hc pcDNA-HBx pcDNA-HBp
Figure 3

Fig. 3A

<table>
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<tr>
<td>GAPDH mRNA</td>
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Fig. 3B

<table>
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<tr>
<td>GAPDH protein</td>
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</tr>
</tbody>
</table>

Fig. 3C

![MAT II activity graph](image)

![HBX](image)  
![GAPDH](image)
Fig. 4A

-951
\[ \text{NF-\(\kappa\)B} \quad \text{CRE} \quad \text{NF-\(\kappa\)B} \quad \text{Luc} \]

548
\[ \text{NF-\(\kappa\)B} \quad \text{CRE} \quad \text{NF-\(\kappa\)B} \quad \text{Luc} \]

-320
\[ \text{NF-\(\kappa\)B} \quad \text{Luc} \]

-108
\[ \text{Luc} \]

Fig. 4B

wild-type
\[ \text{NF-\(\kappa\)B1} \quad \text{CRE} \quad \text{NF-\(\kappa\)B2} \quad \text{Luc} \]

mt-NF-\(\kappa\)B1
\[ \text{NF-\(\kappa\)B1} \quad \text{CRE} \quad \text{NF-\(\kappa\)B2} \quad \text{Luc} \]

mt-CRE
\[ \text{NF-\(\kappa\)B1} \quad \text{CRE} \quad \text{NF-\(\kappa\)B2} \quad \text{Luc} \]

mt-NF-\(\kappa\)B2
\[ \text{NF-\(\kappa\)B1} \quad \text{CRE} \quad \text{NF-\(\kappa\)B2} \quad \text{Luc} \]
**Fig. 5A**

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<td>Specific competition</td>
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**Fig. 5B**

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<td>HBx (µg)</td>
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Figure 6

Fig. 6A

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<tr>
<td>GABDH</td>
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<td>-</td>
<td>-</td>
<td>-</td>
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</table>

MAT2A Activity

0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0

MAT2A Protein

GABDH Protein
**Fig. 6B**

![Graph showing MAT II enzyme activity with data points for various treatments: HBx, MG132, LY294002, U0126, SB203580, GF109203, SP600125, H89, PD098095.](http://www.jbc.org/)

**Fig. 6C**

![Bar graph showing relative luciferase activity for various shRNAs: Control, RIG-I, MAVS, IRAK2, TRAF6, IKKa, IKKβ, IKKi, P50, P65.](http://www.jbc.org/)
Fig. 6D

![Graph showing MAT II Activity with MG123 (µg) on the x-axis and MAT II Activity on the y-axis.]

Fig. 6E

![Graph showing MAT II Activity with MG132 (µM) on the x-axis and MAT II Activity on the y-axis.]

![Image of gel electrophoresis showing MAT2A mRNA and GAPDH mRNA.]

![Bar graph showing Control CREB and shRNA CREB with MAT2A mRNA and GAPDH mRNA.]

![Image of gel electrophoresis showing MAT2A mRNA and GAPDH mRNA.]
Figure 7

Fig. 7A

Fig. 7B

Fig. 7C

Fig. 7D

The level of SAM and SAH (nmol/μg protein)

PC DNA-3.1  PC DNA-MAT2A  PC DNA-HBx  MAT2A-siRNA  PC DNA-HBX+MAT2A-siRNA

Apoptosis rate (%)

0  5  10  15  20  25  30  35  40

PC DNA-3.1  PC DNA-MAT2A  PC DNA-HBx  MAT2A-siRNA  PC DNA-HBX+MAT2A-siRNA

SAM  SAH
pcDNA-HBx (μg)  0  0.25  0.5
MAT1A Protein
MAT2A Protein
MAT2β Protein
GAPDH Protein

The level of SAM and SAH (nmol/μg protein)
High level of SAM controls hepatocyte growth and differentiation as well as sensitivity to liver injury.

Normal liver cells

Low level of SAM inhibits apoptosis and predisposes the liver to develop steatohepatitis, cirrhosis, and ultimately HCC.

HBV-infected liver cells

Switch in gene expression

①Activation
②Activation
THE X PROTEIN OF HEPATITIS B VIRUS INHIBITS APOPTOSIS IN HEPATOMA CELLS THROUGH ENHANCING THE METHIONINE ADENOSYLTRANSFERASE 2A GENE EXPRESSION AND REDUCING S-ADENOSYL-METHIONINE PRODUCTION
Quanyan Liu, Jiwei Chen, Li Liu, Jun Zhang, Dongfeng Wang, Lu Ma, Yueming He, Yingle Liu, Zhisu Liu and Jianguo Wu

J. Biol. Chem. published online January 19, 2011

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